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(54) Title: DNA REPAIR

(57) Abstract

The invention relates to the identification of a polypeptide which shows mismatch binding activity *in vitro* for use in mismatch binding assays with a view to detecting mutations and/or polymorphisms in genetic material.

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DNA Repair

Field of Invention

The invention relates to products and methods for use in identifying mismatched oligonucleotides in at least a fragment of genetic material for use, particularly but not exclusively, in the diagnosis of genetic disease.

The diagnosis of genetic disease using DNA markers linked to disease genes or, increasingly, the direct analysis of the relevant gene for mutations of the DNA sequence is of enormous clinical importance. If it were possible to simplify the detection of DNA sequence mutations a common approach could be adopted for the diagnosis of, or prediction of, predisposition to a wide range of inherited and acquired conditions. The most sensitive method currently available for the detection of mutations is probably that of Chemical Cleavage of Mutations (CCM). This method can theoretically detect all mismatches and small insertions and deletions and provide an approximate location of the mismatch. However, this approach is cumbersome and difficult to use in practice; still requires confirmation by DNA sequencing; and to batch large sample numbers this method would become very time-consuming. New methods are now being investigated based on the use of proof-reading enzymes or repair enzymes where function *in vitro* is the recognition of mismatches.

Just one example of a disease characterised by a high incidence of mutation is hereditary non-polyposis colorectal cancer (HNPCC). This is one of the most common human autosomal dominant diseases. Estimations are that up

to one in two hundred of the population may be heterozygotes for mutant alleles and be predisposed to develop this form of cancer (13). HNPCC kindreds have a high incidence of mutation in tumour micro-satellite sequences when these are compared with the normal repeats occurring in non-malignant tissues from the same individuals (14). Also, cell lines derived from HNPCC tumours are genetically unstable (15). These observations suggest that a possible causative factor for HNPCC is a failure in DNA repair. Indeed, it has now been shown that cancer predisposition in these patients is attributable to defects in any one of four human genes involved in mismatch repair, hMSH-2, hMLH-1, hPMS-1 or hPMS-2 (12, 16-20).

The best studied mismatch repair pathway is the methyl-directed long patch repair pathway in *E. coli* (1), which is involved in increasing the fidelity of replication by specific repair of DNA polymerase incorporation errors. Initiation of heteroduplex repair is dependent on the product of the MutS gene, which binds to base mispairs and loops of up to four unpaired nucleotides. After this, the MutL gene product binds to the DNA-MutS complex, initiating excision of a tract of single-stranded DNA that contains the mismatched residue(s). The repair process is completed by resynthesis of the excised DNA strand and ligation of the remaining nick.

Mutations of MutS and MutL, or their homologues in yeast, lead to increases in the rate of spontaneous mutation resulting in a mutator phenotype (2, 3). The human proteins hMSH-2 and the recently discovered G/T binding protein (GTBP), which are believed to form a heterodimer (hMutSα) are homologs of MutS (4, 5), whereas the human proteins hMLH-1, hPMS-1 and hPMS-2 are analogous to MutL. Mutations in any of these genes are believed to inactivate mismatch repair in man and yield a mutator phenotype

which can destroy the normal functioning of critical genes and lead to tumour formation (6).

MutS has been used in methods involving proof-reading or repair enzymes. Binding of this enzyme to mismatches has been shown to be sensitive and specific (7). However, MutS which has been expressed in bacterial systems and purified is unstable.

The cDNA of hMSH-2 is known (12) furthermore hMSH-2 has been shown to bind to DNA containing nucleotide mismatches *in vitro* (8,9). However, it is difficult to achieve acceptable levels of expression of hMSH-2 in expression systems. Our own work to express hMSH-2 in baculovirus expression systems has not been successful. This is probably a consequence of the overexpression of full length hMSH-2 being deleterious to the growth of the insect virus in cell culture. However, we have shown that fragments of hMSH-2 can be successfully expressed in bacteria and we have identified a domain of the hMSH-2 enzyme which when expressed displays mismatch binding activity *in vitro*. hMS-2 and its homologues are very highly conserved over their carboxy terminal domains. We have examined the domain and we have found that it demonstrates homology to a type A consensus sequence found in many proteins that bind and hydrolyse nucleotides (10). It has been shown that MutS displays a weak ATPase activity in the presence and absence of DNA and genetic alteration of this ATP binding site results in a protein which is defective in mismatch repair. It is therefore notable that we demonstrate herein that the aforementioned domain of hMSH-2 also exhibits ATPase activity.

It is therefore an object of the invention to provide a protein fragment that

shows mismatch binding activity *in vitro*.

According to a first aspect of the invention there is provided an isolated polypeptide showing mismatch nucleotide binding activity *in vitro* said polypeptide comprising at least a part of the C terminal domain of a nucleotide binding protein, or a type A nucleotide binding motif, which domain, or motif, further exhibits ATPase activity.

Reference herein to a type A nucleotide binding motif includes reference to a motif that has been identified following structural studies and shown to comprise a type A sequence including a flexible loop bounded by a β sheet with an α helix on either side (22-26).

Ideally the polypeptide is a part of an enzyme whose functions *in vitro* is the recognition of mismatches such as a proof-reading enzyme or repair enzyme and ideally a C-terminal domain of said enzyme.

More preferably still said polypeptide comprises approximately 300, and more preferably 297, amino acids and ideally the last 270 amino acids of said enzyme.

More preferably further still said polypeptide comprises amino acids 637 to 877 of the protein hMSH-2. Even more preferably still said polypeptide comprises amino acids 664 to 877. More preferably further still said polypeptide comprises amino acids 664 to 805 of hMSH-2.

More preferably yet still said polypeptide comprises the amino acids shown in the alignment sequence of Figure 1, or at least a substantial part thereof,

which part whose function *in vitro* is the recognition of mismatch binding as herein broadly described.

More preferably still said polypeptide comprises the nucleotide binding domain of hMSH-2, or a homologue or analogue thereof, comprising lysine at nucleotide position 675.

According to a second aspect of the invention there is provided an expression system for the manufacture of a protein fragment in accordance with the invention which system comprises a host cell comprising a fragment of DNA encoding the protein fragment of the invention which DNA is functionally coupled to the replication system of the host cell whereby the protein fragment of the invention can be made.

According to a third aspect of the invention there is provided a vector for transforming a host cell whereby the protein fragment of the invention can be made.

According to a fourth aspect of the invention there is provided a method for obtaining the protein fragment of the invention comprising:

a) inserting a fragment of DNA encoding the protein fragment of the invention and any necessary transcriptional/translational control elements into a suitable host cell expression system;

b) providing conditions which favour transcription and translation of said DNA in said host cells;

c) harvesting said host cells;

d) lysing said host cells;

e) collecting the lysate; purifying the fragment.

In each of the above expression systems and methods the host cell is ideally a bacterial host cell.

Although obtaining the protein fragment of the invention has been described with reference to a biological expression system the said fragment may also be synthetically produced.

In yet a further preferred aspect of the invention said protein fragment is provided with a tag for example a C-terminal Flag peptide such as (Asp Tyr Lys Asp Asp Asp Asp Lys). However, any other tag such as a fluorescent marker or radioactive marker may be used.

In the instance where said Flag is used an antibody, ideally monoclonal, which specifically binds to the Flag can be used to identify the fragment.

According to a further aspect of the invention there is provided DNA sequence encoding the protein fragment of the invention.

According to a further aspect of the invention there is provided oligonucleotides for amplifying said DNA.

According to a yet further aspect of the invention there is provided a method

for identifying mismatched oligonucleotides comprising exposing strands of oligonucleotides to the protein fragment of the invention under conditions which promote binding; and determining the amount of binding taking place.

If preferred, the said oligonucleotides can be tagged using, for example, a radio label.

According to a yet further aspect of the invention there is provided a kit for determining mismatch binding comprising at least the protein fragment of the invention.

Ideally said kit comprises a control comprising at least one mismatched binding pair of oligonucleotides and ideally at least one matched complementary binding pair of oligonucleotides.

According to a yet further aspect of the invention there is provided the use of a fragment of a nucleotide binding protein for detection of mismatched complementary oligonucleotide pairs or of mismatches in double-stranded nucleic acid fragments or in double-stranded PCR products.

According to a yet further aspect of the invention there is provided means for regulating the activity of a nucleotide binding protein, or a fragment thereof, comprising the substitution, or deletion, of a critical codon, or amino acid, in the nucleotide binding domain thereof.

Preferably, said substitution or deletion, comprises a manipulation of: the codon encoding the amino acid lysine at codon 675 of hMSH-2, or its equivalent in a homologous or analogous protein; or the corresponding lysine

amino acid, so that lysine is either substituted or deleted in the relevant protein, or a fragment thereof.

An embodiment of the invention will now be described by way of example only with reference to the following figures, materials and methods wherein:

Figure 1 Shows alignment of amino acid sequences of the conserved COOH terminal region of hMSH-2, and MSH-2 and MutS.

Figure 2 Shows how a DNA fragment containing the carboxy terminal domain of hMSH-2 was generated using PCR. This fragment contained amino acids 611 to 852 of the published sequence (ii). The domain was ligated to pFlag.CTC to derive phMSH2.Flag.

Figure 3 Shows analysis of hMSH-2 flag fusion protein.

Transformed *E. coli* were grown at 37°C for 2 hours, the cultures were grown for a further 5 hours (Lane 1), or induced with IPTG (1 mM) and grown for 0 hours (Lane 2), 2 hours (Lane 3) and 5 hours (Lane 4). Extracts were resolved by SDS PAGE, transferred to nitrocellulose, and incubated with M2 monoclonal antibody (IgG_1) to the flag epitope and immune complexes were detected by using rabbit anti mouse immunoglobulin conjugated with horseradish peroxidase. The nitrocellulose membranes were developed in PBS containing 0.02% 1-chloro-4-naphtol and 0.006% hydrogen peroxide.

Figure 4 Shows ATPase analysis of the bacterial fusion. Hydrolysis of various substrate concentrations of [α-^{32}P]ATP by the carboxy terminal domain of hMSH-2 was assayed by thin layer chromatography, and quantified using a scintillation counter.

Figs. 5&5A Show functional analysis of the bacterial fusion protein.

Oligonucleotides containing either a perfect match a selected single mismatch were radiolabelled using primer extension. One pmole of labelled DNA was incubated for 1 hour with: Figure 5 *E. coli* MutS (Lane 1), or protein extracts of Flag (Lane 2), or hMSH-2.Flag (Lane 3-4); or Figure 5A protein extracts of hMSH-2.Flag or Flag. After the incubation period the mixtures were slowly filtered over preset nitrocellulose, washed and bound DNA was detected using autoradiography.

Figs 6&6A Shows quantification of the binding assay.

Figures 6 and 6A correspond to Figures 5 and 5A, respectively.

Radioactive spots on the nitrocellulose filter were excised and quantified using a scintillation counter. The results are shown as counts per minute and variations between three replicated assays are indicated.

Figure 7 Homology between hMSH-2 DNA binding domain and the 'Type A' consensus sequence. Bold type indicates conserved residues between both proteins. The mutants produced are

indicated showing the alteration of each conserved residue.

Figure 8 Analysis of expression of mutant fusion proteins. Coomassie blue stained SDS PAGE gel showing protein extracts analysed 4 hours post induction with 1mM IPTG. Lane 1-2, hMSH-2 domain - uninduced and induced; Lane 3-4, Δ1 - uninduced and induced; Lane 5-6, Δ2 - uninduced and induced; Lane 7-8, Δ3 - uninduced and induced; Lane 9-10, Δ4 - uninduced and induced; Lane 11-12, Δ5 - uninduced and induced, respectively.

Figure 9 ATPase analysis of the mutant bacterial fusion proteins. Hydrolysis of various substrate concentrations of [α-^{32}=P]ATP by the carboxy terminal domain of hMSH-2, pET and Δ1-5 were assayed by thin layer chromatography, and quantified using a scintillation counter.

Figure 10 Functional analysis of the mutant bacterial fusion proteins. Oligonucleotides containing either a perfect match or a range of single mismatches were radiolabelled using polynucleotide kinase. One pmole of labelled DNA was incubated for 1 hour with protein extracts of hMSH-2, pET and Δ1-5. After the incubation period the mixtures were slowly filtered over prewet nitrocellulose. Washed and bound DNA was detected using autoradiography.

Materials and Methods

Construction of a hMSH-2 C-terminal domain expression vector

A DNA fragment containing the cDNA fragment required from hMSH-2 was generated by polymerase chain reaction (PCR) using 10ng of plasmid pBShMSH2 DNA, and 250ng of the oligonucleotides dCCG AAG CTT AGG CAT GCT TGT GTT GAA GTT CAA GAT and dGCG GGA TCC TCT TTC CAG ATA GCA CTT CTT TGC TGC. These oligonucleotides incorporated *Bam*HI and *Hind*III restriction sites respectively, for convenient cloning of the PCR product. They generate a PCR fragment which encodes amino acid 637 to amino acid 877 in the published sequence (11). The reaction was performed with 4 units of Taq polymerase (Promega) in the buffer recommended by the supplier. After 30 cycles (1 minute, 92°C, 1 minute 60°C, 1 minute 72°C), the DNA produced was phenol/chloroform extracted, ethanol precipitated, digested with *Bam*HI and *Hind*III and cloned into the corresponding sites of pFlag.CTC (IBI) to derive phMSH2.Flag. The integrity of the insert was checked by DNA sequencing (data not shown).

Expression of hMSH-2 C-terminal domain as a bacterial fusion protein

The PCR product encoding the 637 to 877 amino acid hMSH-2 domain in the Flag bacterial expression vector (IBI), was used to transform *E. coli* strain DH5α. A fresh overnight culture of transformed *E. coli* was diluted 1 in 20 with LB medium containing ampicillin (100 μg/ml). After growth at 37°C for 2 hours, the culture was induced with IPTG (1 mM) and grown at 37°C for a further 5 hours. The cells were harvested by centrifugation at 3200g for 10 minutes and resuspended in 0.1 volume lysis buffer (100mM Tris-HCl, pH 8.0, 1mM EDTA) and incubated on ice with 3 mg/ml of lysozyme for 30 minutes. The cells were then sonicated and lysed by the addition of Tween 20 lysis buffer (100 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.3 mg/ml phenylmethylsulphonyl fluoride, 0.8 μg/ml pepstatin, 1 mM DTT, 1%

Tween 20). Cellular debris was pelleted by centrifugation at 4,000g.

Detection of fusion protein by Western blot analysis

Protein extracts were mixed with 2x reducing sample buffer (50mM Tris-HCI, pH 6.8, 4% sodium dodecyl sulphate (SDS), 5 mM EDTA, 10% β-mercapthoethanol, 1 mM DTT and 0.01% bromophenol blue). After boiling for 3 minutes, samples were fractionated on a 12% SDS polyacrylamide gel. After electrophoresis the gel was soaked for 10 minutes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (v:v), and 0.1% SDS), and the proteins were transferred to nitrocellulose membranes by electroblotting for 3 hours at 250 mA. After transfer, the membranes were soaked in PBS and incubated for 2 hours in blocking buffer (PBS containing 5% nonfat dry milk). Membranes were incubated with a 1/100 dilution of the M2 monoclonal antibody (IgG_1, IBI), washed with PBS and incubated for 1 hour at 37°C with a 1/1000 dilution of rabbit anti-mouse immunoglobulin conjugated with horseradish peroxidase in blocking buffer. After five washes with PBS the nitrocellulose membranes were developed in PBS containing 0.02% 1-chloro-4-naphthol and 0.006% hydrogen peroxide.

ATPase Assay

The assay was performed at 37°C in 20 mM Tris-HCI pH 7.6, 0.5 mM $CaCl_2$, 5mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 0.1 mM EDTA and 150 ng of hMSH-2 domain. Assays were performed using 2, 2.5, 3.3, 5 and 10 μM ATP. Hydrolysis of [α-^{32}P]ATP by the carboxy terminal domain of hMSH-2 was assayed by thin layer chromatography. The radioactive counts for ATP and its hydrolysis products were quantified using a scintillation

counter (Packard).

Functional binding assay

Mismatch binding was detected by a nitrocellulose binding assay of labelled oligonucleotides followed by autoradiography. Oligonucleotides (dCGG ATC CGG AXG TCA TGG AAT TCC and dGGA ATT CCA TGA CXT CCG GAT CCG) were synthesised and annealed to produce either a perfect matched double-stranded molecule or a single G:T mismatch (position shown in bold type). Oligonucleotides were mixed to a final concentration of 100 pmole/μl each in 100 μl STM (100 mM NaCl, 10 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 5 mM DTT) heated to 95°C and cooled to 25°C over 2 hours. The annealed products were then stored in 50% glycerol at -20°C until required. End-labelling of double-stranded DNA (100 pmole) in STM buffer was by polynucleotide kinase. After incubating at 20°C for 10 minutes the unincorporated label was removed using a Sephadex NAP 5 column. The labelled DNA was diluted to 0.2 pmole/μl. The binding assay used 1 pmole of DNA with 150 ng hMSH-2 domain in a total volume of 10 μl. After 1 hour on ice the mixture was slowly filtered over pure prewetted nitrocellulose (Millipore, 0.45μm) and washed in STM buffer. The filter was then allowed to air dry and bound DNA was detected by autoradiography. Bound material was quantified using a scintillation counter (Packard).

Construction of mutant hMSH-2 nucleotide binding domain expression vectors

DNA fragments expressing the C-terminal DNA binding domain sufficient to bind specific mismatched oligonucleotides and mutants 1-5 shown in Figure 7 were generated by polymerase chain reaction (PCR) using 10ng of

plasmid pBShMSH-2 DNA, and 250ng of the following forward primers dCCG GGA TCC TTC CAC ATC ATT ACT GGC CCC AAT ATG GGA GGT AAA TCA; dCCG GGA TCC TTC CAC ATC **GGT** ACT GGC CCC AAT ATG GGA GGT AAA TCA; dCCG GGA TCCTTC CAC ATC ATT ACT GCC CCC AAT ATG GGA GGT AAA TCA; dCCG GGA TCC TTC CAC ATC ATT ACT GGC CCC AAT ATG GGA **GCT** AAA TCA; dCCG GGA TCC TTC CAC ATC ATT ACT GGC CCC AAT ATG GGA GGT **GCA** TCA; dCCG GGA TCC TTC CAC ATC ATT ACT GGC CCC AAT ATG GGA GGT AAA **GCA** and the reverse primer dGCG GGA TCC TCT TTC CAG ATA GCA CTT CTT TGC TGC (changes shown in bold type). These oligonucletoides incorporated *Bam*HI restriction sites for convenient cloning of the PCR products. The reaction was performed with 4 units of *Pfu* DNA Polymerase (Stratagene) in the buffer recommended by the supplier. After 30 cycles (1 min, 92°C, 1 min 60°C, 1 min 72°C), the DNA produced was phenol/chloroform extracted, ethanol precipitated, digested with *Bam*HI and cloned into the corresponding site of pET21a (Novagen) to derive pEThMSH-2 and PETΔ1-5 respectively. The integrity of each insert was confirmed by DNA sequencing (data not shown).

Production of hMSH-2 nucleotide binding domain mutants as bacterial fusion proteins

The wild type and mutant proteins encoding the amino acid 663-877 hMSH-2 domain in the pET bacterial expression vector, were used to transform *E. coli* strain BL21(DE3). A fresh overnight culture of transformed *E. coli* was diluted 1 in 20 with LB medium containing ampicillin (100 μg/ml). After growth at 37°C for 2 hours, the culture was induced with IPTG (1 mM) and grown at 37°C for a further 5 hours. The cells were harvested by

centrifugation at 3200g for 10 minutes and resuspended in 0.1 volume lysis buffer (100mM Tris-HCl, pH 8.0, 1mM EDTA) and incubated on ice with 3 mg/ml of lysozyme for 30 minutes. The cells were than sonicated and lysed by the addition of Tween 20 lysis buffer (100 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.3 mg/ml phenylmethylsulphonyl fluoride, 0.8 µ.0, 200 mM NaCl, 1 mM EDTA, 0.3 mg/ml phenylmethylsulphonyl fluoride, 0.8 µg/ml pepstatin, 1 mM DTT, 1% Tween 20). Cellular debris was pelleted by centrifugation at 4,000g.

Detection of fusion protein by SDS-PAGE

Protein extracts were mixed with 2x reducing sample buffer (50mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulphate (SDS), 5mM EDTA, 10% β-mercapthoethanol, 1 mM DTT and 0.01% bromophenol blue). After boiling for 3 minutes, samples were fractionated on a 12% SDS polyacrylamide gel. Following electrophoresis the gel was stained with Coomassie blue solution (25% v/v isopropyl alcohol, 10% v/v acetic acid and 0.25% w/v Coomassie blue).

ATPase assay

The assay was performed at 37°C in 20mM Tris-HCl, pH 7.6, 0.5 mM $CaCl_2$, 5mM $MgCl_2$, 1 mM DTT, 100 µg/ml BSA, 0.1 mM EDTA with 150 ng of wild type or mutant hMSH-2 domains. Assays were performed using 2, 2.5, 3.3, 5 and 10 µM ATP. Hydrolysis of [α-^{32}P]ATP by the wild type and each mutant carboxy terminal domain was assayed by thin layer chromatography. The radioactive counts for ATP and its hydrolysis products were quantified using a scintillation counter (Packard).

Functional binding assay

Mismatch recognition was detected by a nitrocellulose binding assay of labelled oligonucleotides followed by autoradiography as described previously (1). Briefly, oligonucleotides (dCGG ATC CGG AXG TCA TGG AAT TCC and dGGA ATT CCA TXA CAT CCG GAT CCG) were annealed to produce either a perfect matched double-stranded molecule or a single mismatch (position shown in bold type). Oligonucleotides were mixed to a final concentration of 100 pmole/µl each in 100 µl STM (100 mM NaCl, 10 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 5 mM DTT (heated to 95°C and cooled to 25°C over 2 hours. End-labelling of double-stranded DNA (100 pmole) in STM buffer was performed with polynucleotide kinase. After incubation at 20°C for 10 minutes the unincorporated label was removed using a Sephadex NAP 5 column. The labelled DNA was diluted to 0.2 pmole/µl. The binding assay used 1 pmole of DNA with 150 ng of wild type or each mutant hMSH-2 domain in a total volume of 10 µl. After 1 hour on ice the mixture was slowly filtered over pure prewetted nitrocellulose (Millipore, 0.45µm) and washed in STM buffer. The filter was then allowed to air dry and bound DNA was detected by autoradiography.

Results

PCR amplification and Cloning

Protein sequence alignments of hMSH-2 and its homologues, MutS, MSH-2 and GTBP revealed a highly conserved region at the COOH terminus (Figure 1). This region contains a type A nucleotide binding site consensus sequence. A 720 bp fragment was amplified using PCR, incorporating *Bam*HI and

*Hind*III restriction sites for convenient cloning. This fragment of the hMSH-2 cDNA sequence encodes amino acid residues 637 to 877. The PCR product was ligated to pFlag.CTC, in phase with respect to the ATG translational start codon immediately upstream of the multiple cloning site (MCS) and also in frame with the C-terminal coding sequence immediately downstream of the MCS to ensure proper fusion to the C-terminal Flag peptide (Asp Tyr Lys Asp Asp Asp Asp Lys). [Figure 2]

Expression of the hMSH-2 C-terminal domain

The hMSH-2 domain was thus cloned into the bacterial expression vector Flag (IBI). Expression of the hMSH-2 Flag fusion protein resulted in a 30 kDa species detected by Western blot analysis on SDS-PAGE (Figure 3). The anti-Flag M2 monoclonal (IgG1) mouse antibody (IBI) was used to specifically bind to the eight amino acid Flag peptide, which identified the 249 amino acid recombinant protein comprising the hMSH-2 domain (containing a type A nucleotide binding site consensus sequence) coupled to the Flag peptide at its carboxy terminus.

ATPase Analysis of Bacterial Fusion Protein

The Walkers A-type nucleotide binding motif conserved in MutS proteins has been shown to have ATPase activity (21). In order to determine whether the carboxy terminal domain of hMSH-2 hydrolyses ATP to ADP and Pi, [α^{32}P]ATP was incubated with the fusion protein and separated using TLC. To determine K_m and k_{cat} values of the hMSH-2 domain, ATPase activity was measured in the presence of various concentrations of ATP (Fig. 4). At 37°C the K_m and k_{cat} were calculated to be 6.6 μM and 0.5 s^{-1}, respectively. In a

control experiment, nonenzymatic hydrolysis of ATP in the absence of the expressed domain was less than 5%.

Functional analysis of the bacterial fusion protein

A mismatch binding assay was developed to measure the hMSH-2 C-terminal domain's activity. Mismatch binding was detected by nitrocellulose binding of labelled oligonucleotides containing a mismatch at position 11 within the context of a double-stranded 24mer oligonucleotide pair. The binding of the hMSH-2 domain to an G-T mismatch containing oligonucleotides is shown in Figure 5. The binding of the hMSH-2 domain to a range of mismatch containing oligonucleotides is shown in Figures 5A. Radiolabelled oligonucleotides containing a perfect match or a single mismatch were incubated with purified MutS or protein extracts containing hMSH-2.Flag or Flag alone. The binding of proteins to mismatched or matched oligonucleotides was quantified using a scintillation counter (Figures 6 and 6A). In Figure 6 it can be seen that the MutS and hMSH-2 domain selectively bound to the oligonucleotides containing the mismatch, but not the perfectly matched oligonucleotides. In Figure 6A it can be seen that hMSH-2 domain selectively bound to the oligonucleotides containing all possible mismatches, apart from C/C and A/A mismatches. The flag control bound to no oligonucleotide, showing the hMSH-2 domain alone is sufficient to bind oligonucleotides containing a G/T mismatch.

PCR amplification and cloning

A fragment of the hMSH-2 cDNA sequence which encodes amino acid residues 637 to 877 has been shown to bind oligonucleotides containing

mismatches (27). In order to determine which specific residues are important in this domain, mutant proteins have been produced which alter specific residues within the putative nucleotide binding region (Fig. 7). DNA fragments which encode the nucleotide binding domain of hMSH-2 were amplified using PCR, incorporating *Bam*HI restriction sites for convenient cloning. Each product was ligated to the expression vector pET21a, in phase with respect to the ATG translational start codon immediately upstream of the multiple cloning site (MCS) and also in frame with the C-terminal coding sequence immediately downstream of the MCS to ensure proper fusion to the C-terminal HisTag.

Expression of the hMSH-2 nucleotide binding domain mutants

To confirm their integrity, each mutant hMSH-2 nucleotide binding domain was cloned into the bacterial expression vector pET21a. Expression of the mutant hMSH-2 fusion proteins resulted in 30 kDa species detected using SDS-PAGE comprising the hMSH-2 domain (containing a type A nucleotide binding site consensus sequence) coupled to the HisTag peptide at its carboxy terminus. (Fig. 8). We designate these mutant fusion proteins Δ1 to Δ5. All mutant proteins were expressed at comparable levels to the wild type fusion protein.

ATPase analysis of mutant fusion proteins

It has been shown that the carboxy terminal domain of hMSH02 contains ATPase activity (27). In order to determine whether these mutants hydrolyse ATP to ADP and Pi, [α^{32}P]ATP was incubated with each mutant fusion protein and separated using TLC. To determine K_m and k_{cat} values for the

mutants, ATPase activity was measured in the presence of various concentrations of ATP (Fig. 9). The results show that Δ1, Δ2, Δ3 have limited effects on the ATPase activity of the domain. Wild type K_m and k_{cat} values were 8.33μM and 0.55 S^{-1}, respectively, compared to Δ2 values of 5.88 μM and 0.633 S^{-1}, respectively. However, Δ5 had a reduced activity with K_m and k_{cat} values of 3.6 μM and 0.65 S^{-1}, and Δ4 which alters the codon 675 from a lysine to an alanine has a marked effect upon ATP hydrolysis, effectively reducing it to zero. In a control experiment, nonenzymatic hydrolysis of ATP in the absence of the wild type expressed domain was less than 5%.

Functional analysis of the mutant fusion proteins

A mismatch binding assay was developed to measure the hMSH-2 C-terminal domains activity (27). Mismatch recognition was detected by nitrocellulose binding of labelled oligonucleotides containing a mismatch at position 11 within the context of a double-stranded 24-mer oligonucleotide pair. We found that the wild type C-terminal domain of hMSH-2 selectively bound all specific mismatches apart from A/A and C/C, in agreement with results described previously (27). The pET control did not bind to any labelled oligonucleotide pair. Applying this assay to the mutant proteins, we found that Δ1, Δ2 and Δ3 bound the same specific mismatches as the wild type domain albeit to a somewhat lesser extent. This may be due to the amino acid substitutions reducing recognition of the mismatches or a reduced affinity of these proteins once bound to a mismatch resulting in separation from the mismatch in the washing procedures of the assay. Δ5 which alters Ser 676 to an Ala has further reduced affinity for these mismatches and Δ4 was found to have no selective binding to any of the specific mismatches

(Fig. 10).

Discussion

hMSH-2 and its homologues around the domain of the type A nucleotide binding motif, bind mismatched oligonucleotides. Herein we have expressed this domain as a bacterial fusion protein and shown that mismatch-containing oligonucleotides are selectively bound.

The inability of Δ4, which alters the codon 675 from a lysine to an alanine, to identify DNA containing mismatches suggests that this Lys 675 residue is important for the binding function. It is unlikely that the mutation alters the structure of this domain significantly so as to reduce stability in *E. coli* as the expression level of this mutant is comparable to that of wild type hMSH-2 nucleotide binding domain. Thus the deficiency is not due to a gross structural instability. At present the role of Lys 675 within the nucleotide binding site is hMSH-2 is not known. However, similar motifs in other proteins have been analysed. Structural studies have shown that a 'type A' sequence is a flexible loop bounded by a β sheet with an α helix on either side (22-26). This flexible loop allows the protein to undergo conformational change, thus controlling the accessibility of substrate binding or binding site affinities (27). Further studies have shown that an analogous lysine plays an important role in ATP-dependent function of these proteins (27-28).

The key role of Lys 675 is also emphasised by the fact that mutations at residues 666, 668 and indeed 674 (the residue next to the critical lys residue) have minimal effects on mismatch recognition and ATPase activity. Furthermore, mutation of the conserved Ser 676, the residue immediately C-

terminal of Lys 675 still provides a protein with retains 40% of its normal activity. These observations suggest that structural factors alone may not fully explain the importance of Lys 675 and perhaps this basic cationic residue is involved more directly, for example in recognising the phosphate backbone at the mismatch point in mispaired DNA.

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CLAIMS

1. An isolated polypeptide showing mismatch nucleotide binding activity *in vitro*, said polypeptide comprising at least a part of the C terminal domain of a nucleotide binding protein, or a type A nucleotide binding motif, which domain, or motif, further exhibits ATPase activity.

2. A polypeptide according to claim 1 wherein said enzyme is hMSH-2 or a homologue or analogue thereof.

3. A polypeptide according to claims 1 or 2 wherein said fragment comprises no more than the last 300 amino acids of said enzyme.

4. A polypeptide according to claim 3 wherein said fragment comprises no more than the last 270 amino acids of said enzyme.

5. A polypeptide according to any preceding claim which comprises amino acids 637-877 of the protein hMSH-2.

6. A polypeptide according to claim 2 wherein said fragment comprises amino acids 644-877 of the protein hMSH-2.

7. A polypeptide according to claim 2 wherein said fragment comprises amino acids 664-805 of the protein hMSH-2.

8. A polypeptide according to any preceding claim wherein said polypeptide is provided with a tag so as to enable the binding of same to be detected.

9. A polypeptide according to claim 8 wherein said tag is a C-terminal flag peptide.

10. A polypeptide according to claim 9 wherein said tag further includes an antibody adapted to bind to said C-terminal flag peptide.

11. An expression system for the manufacture of a polypeptide according to any of the proceeding claims which system comprises a host cell including a fragment of DNA encoding the polypeptide according to any of the preceding claims which DNA is functionally coupled to the replication system of the host cell whereby said polypeptide can be made.

12. A vector for transforming a host cell whereby the polypeptide according to any one of claims 1-10 of the invention can be made.

13. A method for obtaining a polypeptide according to claims 1 to 10 comprising;

a) inserting a fragment of DNA encoding the said polypeptide and any necessary transcriptional/translational control elements into a suitable host cell expression system;

b) providing conditions which favour transcription and translation of said DNA in said host cell;

c) harvesting said host cells;

d) lysing said host cells;

e) collecting the lysates; and

f) purifying the polypeptide fragment.

14. A DNA sequence encoding the polypeptide according to any one of claims 1-10.

15. Oligonucleotides for amplifying the DNA encoding the polypeptide according to anyone of claims 1-10.

16. A method for identifying mismatched oligonucleotides comprising exposing strands of oligonucleotides to the polypeptide of any one of claims 1-10 under conditions which promote binding and determining the amount of binding taking place.

17. A kit for determining mismatch binding comprising at least a polypeptide according to any one of claims 1-10.

18. A kit according to claim 17 which further comprises a control including at least one mismatch binding pair of oligonucleotides.

19. A kit according to claims 17 or 18 comprising at least one matched complementary binding pair of oligonucleotides.

20. The use of a polypeptide according to any one of claims 1-10 for detection of mismatched complementary oligonucleotide pairs or of mismatches in double-stranded nucleic acid fragments or in double-stranded PCR products.

21. Means for regulating the activity of a nucleotide binding protein, or a fragment thereof, comprising the substitution, or deletion, of a critical codon, or amino acid, in the nucleotide binding domain thereof.

22. Means according to claim 21 wherein said regulation comprises a manipulation of: the codon encoding the amino acid lysine at codon 675 of hMSH-2, or its equivalent in a homologous or analogous protein, or the corresponding lysine amino acid, so that lysine is either substituted or deleted in the relevant protein, or a fragment thereof.

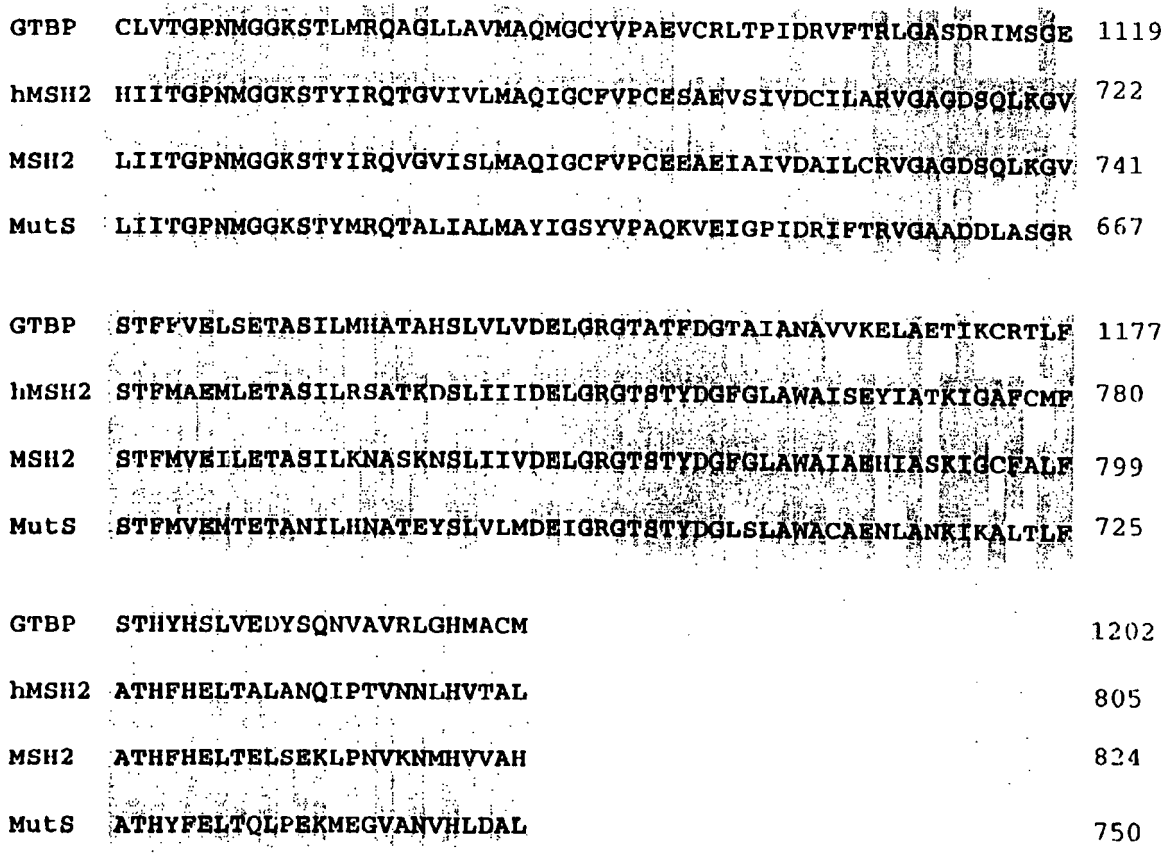

Figure 1. Alignment of amino acid sequences of the conserved COOH terminal region of GTBP, hMSH2, MSH2 and MutS

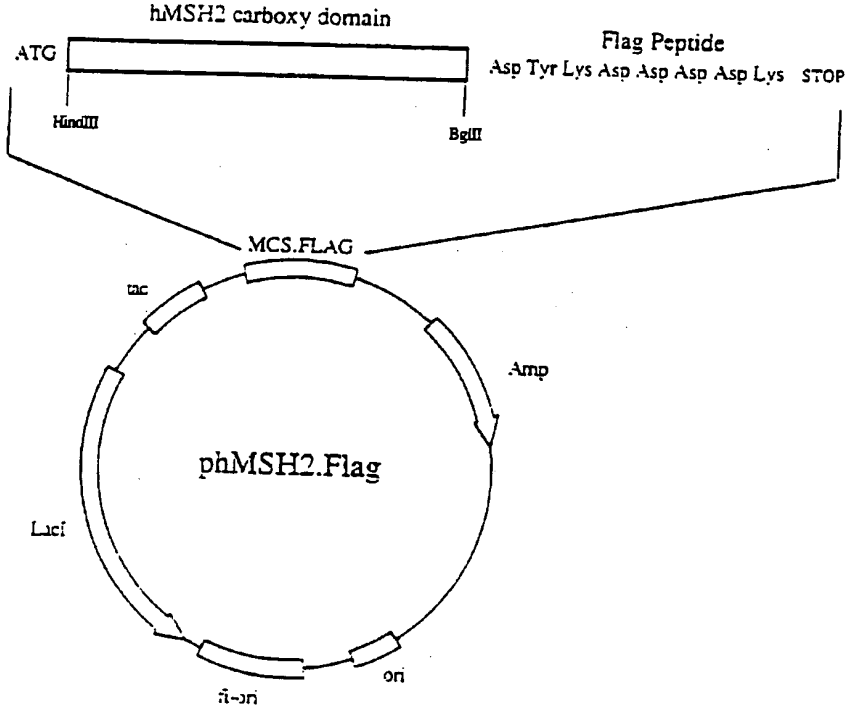

Figure 2. A DNA fragment containing the carboxy terminal domain of hMSH-2 was generated using PCR. This fragment contained amino acids 511 to 852 of the published sequence. The domain was ligated to pFlag.CTC to derive phMSH2.Flag.

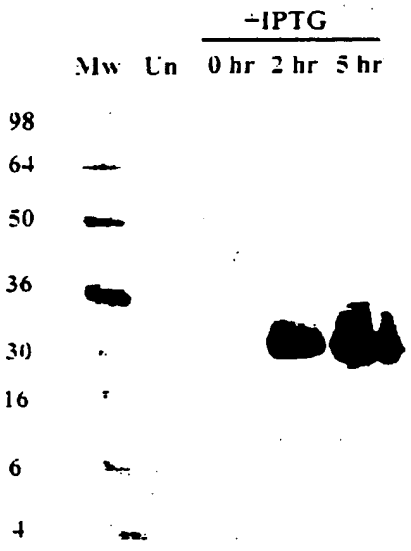

Figure 3

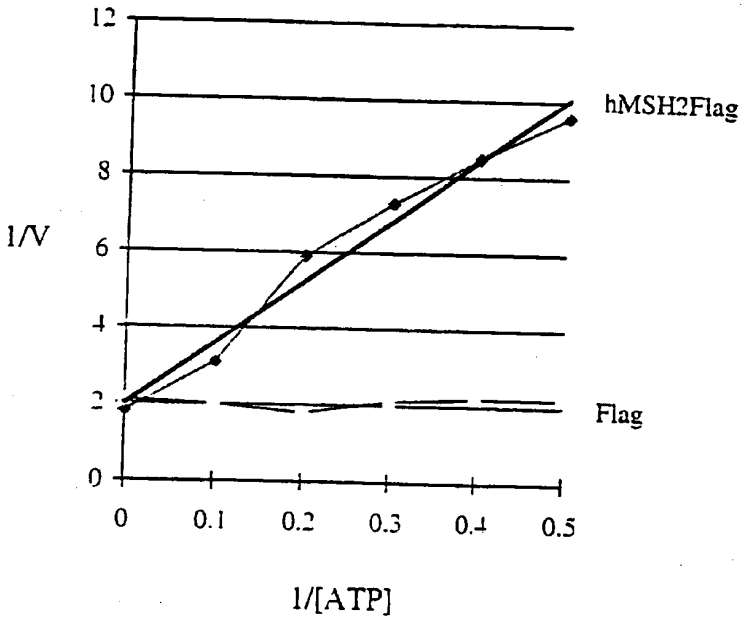

Figure 4

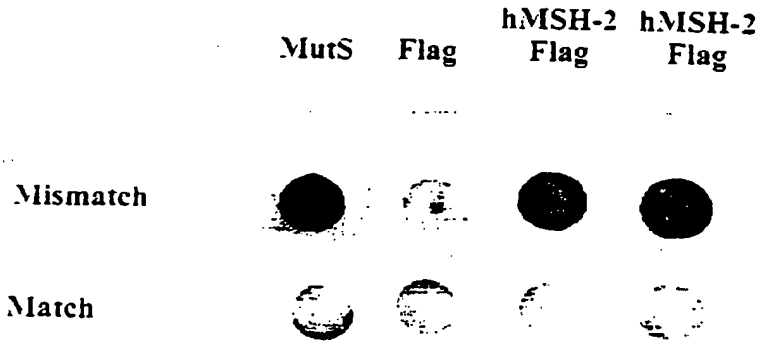

Figure 5.

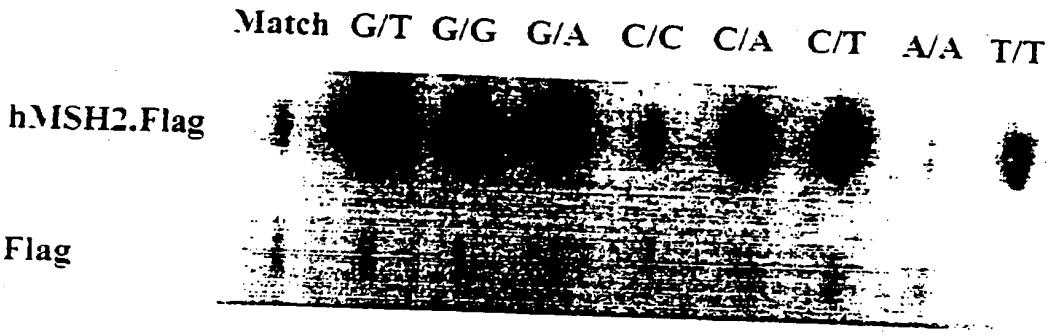

Figure 5A

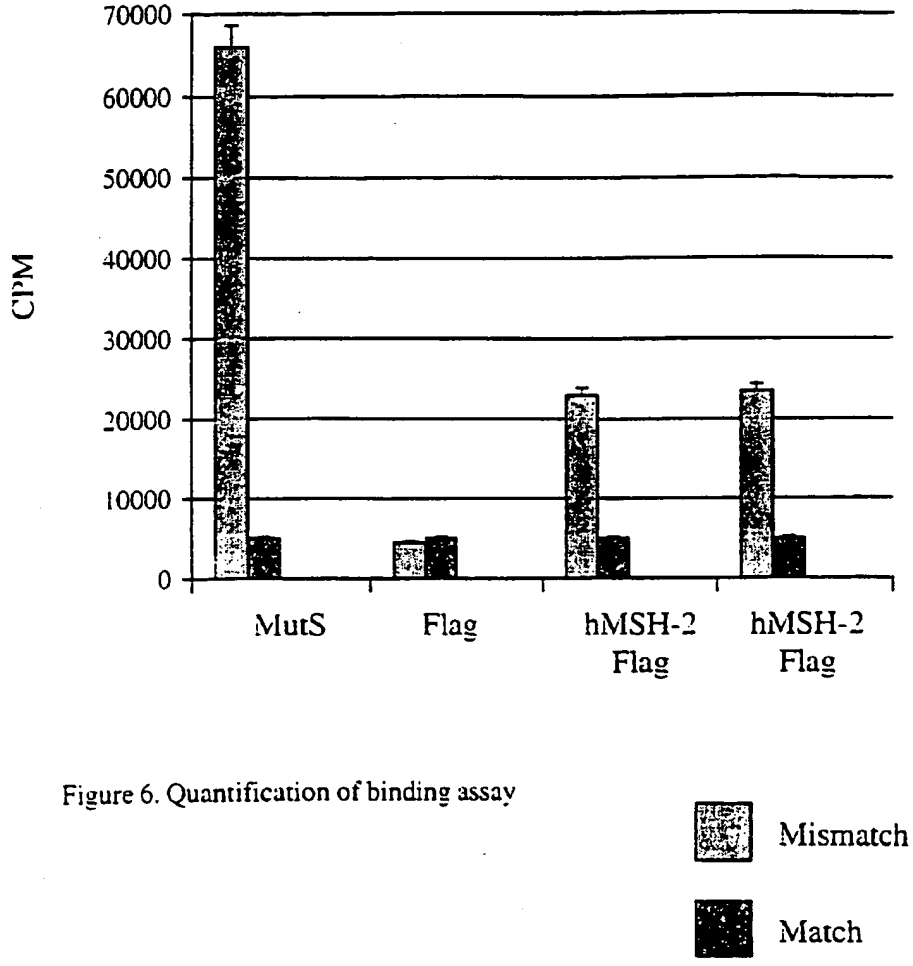

Figure 6. Quantification of binding assay

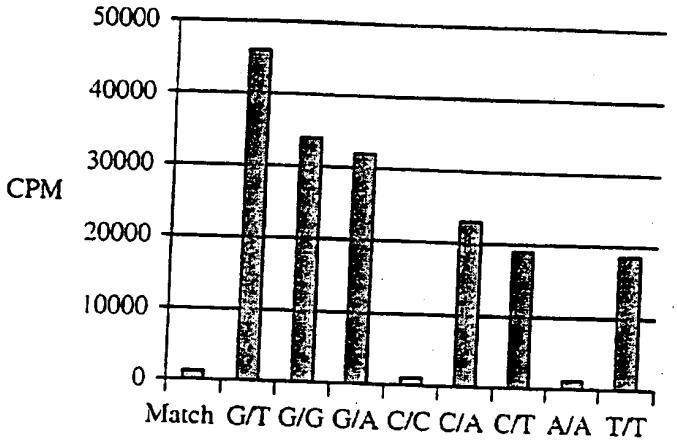
50000
40000
30000
CPM
20000
10000
0
Match G/T G/G G/A C/C C/A C/T A/A T/T

Figure 6A

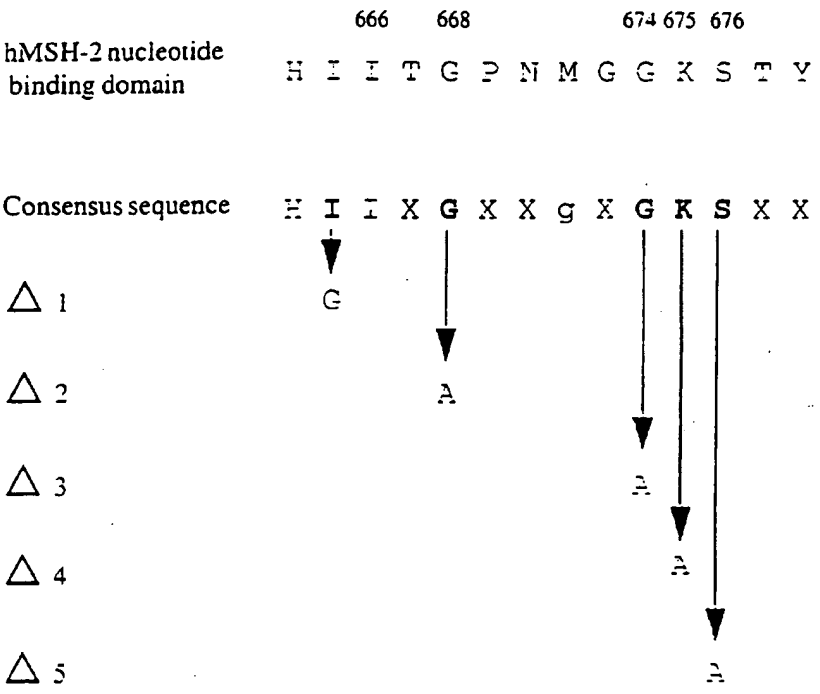
666
668
674 675 676
hMSH-2 nucleotide binding domain
H I I T G P N M G G K S T Y
Consensus sequence
H I I X G X X g X G K S X X
Δ 1
G
Δ 2
A
Δ 3
A
Δ 4
A
Δ 5
A

Figure 7

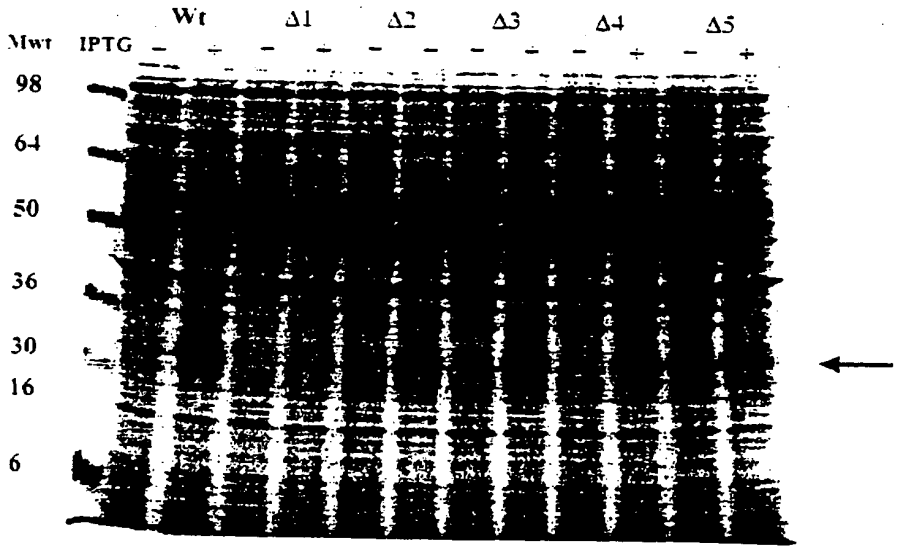

Figure 8

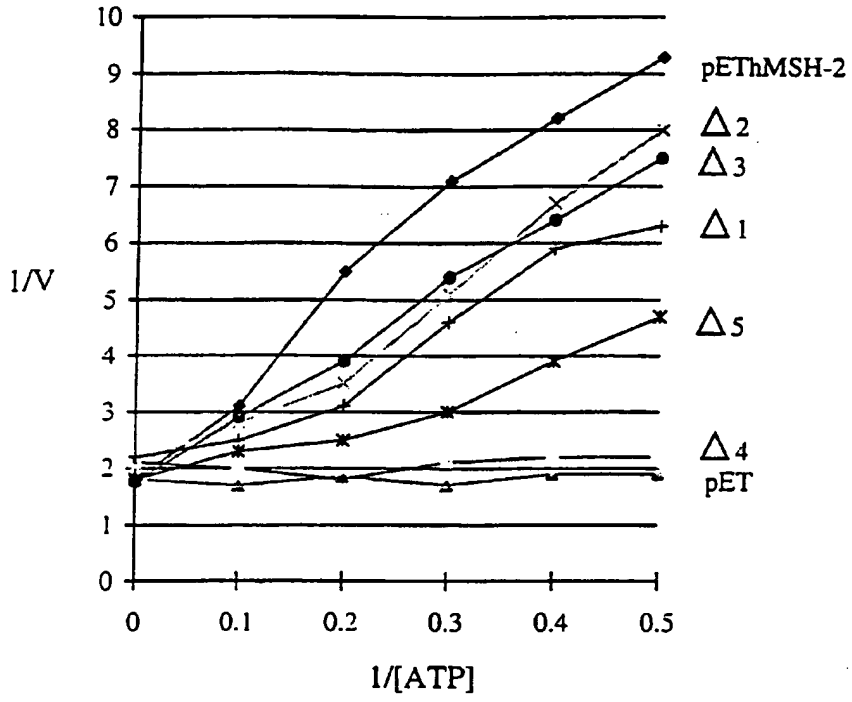
10
9
8
7
6
5
4
3
2
1
0
1/V
0
0.1
0.2
0.3
0.4
0.5
1/[ATP]
pEThMSH-2
Δ2
Δ3
Δ1
Δ5
Δ4
pET

Figure 9

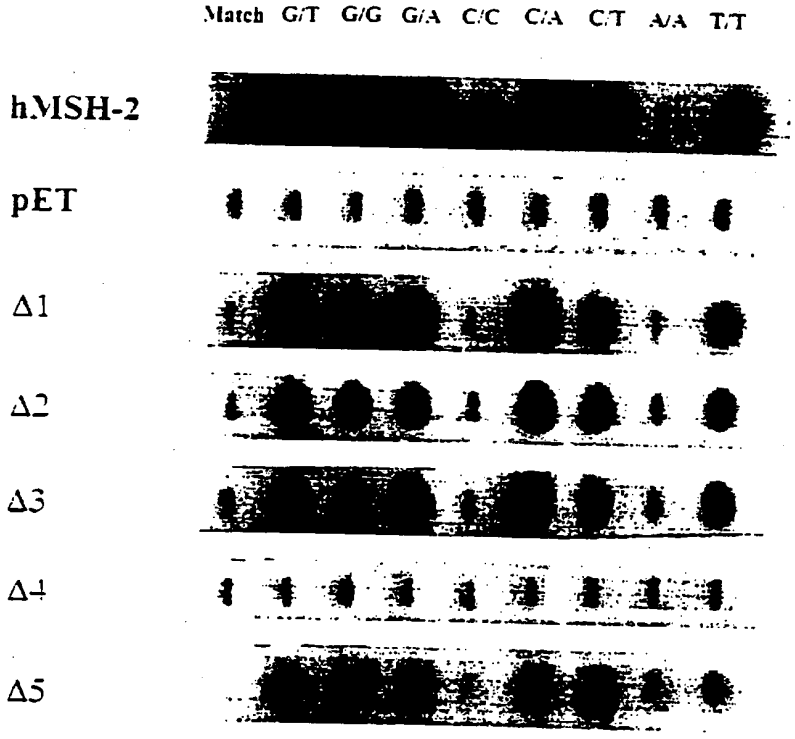

Figure 10

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 96/02595

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/00 C12N15/52 C12N15/63 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BIOCHEMICAL BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 225, 1996, pages 289-295, XP002027431 A. WHITEHOUSE ET AL.: "A carboxy terminal domain of the hMSH2 gene product is sufficient for binding specific mismatched oligonucleotides " *see the whole article* ---	1-22
X	CELL, vol. 81, 1995, pages 1013-1020, XP002027432 S. LEE ET AL.: "p53 and its 14kDa C-terminal domain recognizes primary DNA damage in the form of insertion/deletion mismatches" *see the whole article* ---	1,8-20

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[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
12 March 1997	01. 04. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Marie, A

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF NATIONAL ACADEMY OF SCIENCES USA, vol. 92, 1995, pages 5729-5733, XP002027433 J. H. BAYLE ET AL.: "The carboxy-terminal domain of the p53 protein regulates sequence-speific DNA binding through its nonspecific nucleic acid-binding activity" *see the whole article* ---	1,8-20
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 47, 1994, pages 29993-29997, XP002027434 NAI-WEN CHI ET AL.: "The effect of DNA mismatches on the ATPase activity of MSH1, a protein in yeast mitochondria that recognizes DNA mismatches" *see the whole article* ---	1,8-20
X	EMBO JOURNAL, vol. 10, no. 9, 1991, pages 2707-2715, XP002027435 L.T. HABER ET AL.: "Altering the conserved nucleotide binding motif in the S.typhimurium MutS mismatch repair protein affects both its ATPase and mismatch binding activities" *see the whole article* ---	1,8-20
X	SCIENCE, vol. 266, 1994, pages 1403-1405, XP002027436 R. FISHEL ET AL.: "Binding of mismatched microasatellite DNA sequences by the human MSH2 protein" *see the whole article* ---	1,8-20
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 33, 1992, pages 23876-23882, XP000615520 M.J. HUGHES ET AL.: "The purification of a human mismatch-binding protein and identification of its associated ATPase and helicase activities" *see the whole article* ---	1,8-20
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 23, 1995, pages 14085-14093, XP000651087 S. SHARMA ET AL.: "Cloning, overexpression, purification and characterization of the carboxy-terminal nucleotide binding domain of the P-glycoprotein" *see the whole article* -----	1,8-20

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